LZI Fentanyl (Semi-Quantitative) Enzyme Immunoassay

REF 0550 (100/37.5 mL R_1/R_2 Kit)
0551 (1000/375 mL R_1/R_2 Kit) 2ºC 8ºC

For Forensic Use Only

Lin-Zhi International, Inc.

Intended Use

The LZI Fentanyl (Semi-Quantitative) Enzyme Immunoassay is intended for the semi-quantitative determination of norfentanyl in human urine at the cutoff value of 5 ng/mL when calibrated against norfentanyl. The assay is designed for use with a number of automated clinical chemistry analyzers. This is a non-FDA approved assay for Forensic Use Only and as such should not be repackaged for *in vitro* diagnostic use.

The assay provides only a preliminary analytical result. A more specific alternative chemical method (e.g., gas or liquid chromatography and mass spectrometry) must be used in order to obtain a confirmed analytical result. (1, 2). Clinical consideration and professional judgment should be exercised with any drug of abuse test result, particularly when the preliminary test result is positive.

Summary and Explanation of Test

Fentanyl is an important opioid analgesic used widely in surgical operations and is a controlled substance (1). Fentanyl is most commonly encountered in the form of patches applied to the skin, as "lollipops" which can be dissolved in the mouth through the mucous membrane, or can be administered intravenously. It is 50-100 times stronger than morphine (2, 3) and cases of fentanyl abuse via intravenous injection, inhalation, oral, or nasal applications have been previously reported (4). Fentanyl is used in the treatment of acute and chronic pain, usually in patients who no longer respond to high doses of less potent opioids such as morphine or oxycodone. Due to its potency and wide availability as a prescribed drug, fentanyl has been abused and misused by health professionals, pain management patients, and recreational abusers (5).

Due to its short elimination half-life and approximately 90 % metabolism, fentanyl is difficult to detect in urine (6). Fentanyl undergoes extensive hepatic biotransformation to metabolites coming from hydrolysis, N-dealkylation, or hydroxylation reactions (7). In an intravenous dose of fentanyl, up to 85 % is excreted in urine over a three to four day period with 0.4-6 % eliminated as unchanged fentanyl and 26-55 % eliminated as the norfentanyl metabolite (8).

Fentanyl analogs also have high potency analgesic activities. Numerous reports have been published with modified fentanyl-related compounds abused as designer drugs (9-11).

Other recently available fentanyl analogs associated with abuse and severe intoxication include butyryl fentanyl and 4-fluorobutyryl fentanyl (12-16).

Assay Principle

The LZI Fentanyl (Semi-Quantitative) Enzyme Immunoassay is a homogeneous enzyme immunoassay ready-to-use liquid reagent. The assay is based on competition between drug in the sample and drug labeled with the enzyme glucose-6-phosphate dehydrogenase (G6PDH) for a fixed amount of antibody in the reagent (17). Enzyme activity decreases upon binding to the antibody, and the drug concentration in the sample is measured in terms of enzyme activity. In the absence of drug in the sample, fentanyl-labeled G6PDH conjugate is bound to antibody, and the enzyme activity is inhibited. On the other hand, when drug is present in the sample, antibody would bind to free drug; the unbound fentanyl-labeled G6PDH then exhibits its maximal enzyme activity. Active enzyme converts nicotinamide adenine dinucleotide (NAD) to NADH, resulting in an absorbance change that can be measured spectrophotometrically at 340 nm.

Reagents Provided

Antibody/Substrate Reagent (R_1): Contains a mouse monoclonal anti-fentanyl antibody, glucose-6-phosphate (G6P), nicotinamide adenine dinucleotide (NAD), stabilizers, and sodium azide (0.09 %) as a preservative.

Enzyme-drug Conjugate Reagent (R_2): Contains glucose-6-phosphate dehydrogenase (G6PDH) labeled with fentanyl in buffer with sodium azide (0.09 %) as a preservative.

Calibrators and Controls*

**Calibrators and Controls are sold separately and contain negative human urine with sodium azide as a preservative.*

NORFENTANYL (Semi-Quantitative) Calibrators	REF
Negative Calibrator	0001
Low Calibrator: Contains 2.5 ng/mL norfentanyl	0552
Cutoff Calibrator: Contains 5 ng/mL norfentanyl	0553
Intermediate Calibrator: Contains 10 ng/mL norfentanyl	0554
High Calibrator: Contains 20 ng/mL norfentanyl	0555

NORFENTANYL (Semi-Quantitative) Controls	REF
Level 1 Control: Contains 3.75 ng/mL norfentanyl	0557
Level 2 Control: Contains 6.25 ng/mL norfentanyl	0558

Precautions and Warning

- *This test is for Forensic Use Only. This test should not be re-packaged for in vitro diagnostic use.*
- *Harmful if swallowed.*
- *Reagent contains sodium azide as a preservative, which may form explosive compounds in metal drain lines. When disposing such reagents or wastes, always flush with a large volume of water to prevent azide build-up. See National Institute for Occupational Safety and Health Bulletin: Explosive Azide Hazards (18).*
- *Do not use the reagents beyond their expiration dates.*

Reagent Preparation and Storage

The reagents are ready-to-use. No reagent preparation is required. All assay components should be refrigerated at 2-8ºC when not in use.

Specimen Collection and Handling

Use fresh urine specimens for the test. If the sample cannot be analyzed immediately, it may be refrigerated at 2-8ºC for up to four weeks (19) or at room temperature for up to four weeks (19, 20). For longer storage, keep sample frozen at -20ºC and then thaw before use. Studies have shown norfentanyl samples in urine are stable at -20ºC for up to six months (21). Sample stability claims were established by experimental data by the manufacturer or based on reference literature and only for the temperatures/time frames as stated in the method sheet. It is the responsibility of the individual laboratory to use all available references and/or its own studies to determine specific stability criteria for its laboratory.

Samples should be equilibrated to room temperature (18-25ºC) for testing. Samples with high turbidity should be centrifuged before analysis. Adulteration may cause erroneous results. If sample adulteration is suspected, obtain a new sample and both samples should be forwarded to a laboratory for testing.

Handle all urine specimens as if they are potentially infectious.

Instrument

Clinical chemistry analyzers capable of maintaining a constant temperature, pipetting sample, mixing reagents, measuring enzyme rates at 340 nm and timing the reaction accurately can be used to perform this homogeneous immunoassay.

Performance characteristics presented in this package insert have been validated on the Beckman Coulter®AU680.

Assay Procedure

Typical assay parameters used for the Beckman Coulter AU680 analyzer include a 15 µL sample, 120 µL of antibody reagent (R_1), 45 µL of enzyme conjugate reagent (R_2), 10 µL dilution following addition of R_2 in 37ºC incubation temperature, 14-19 reading points, and 340 nm primary wavelength. Additional washing steps are required, reference analyzer specific parameter sheet.

For semi-quantitative analysis, use all five calibrators.

Recalibration should be performed after reagent bottle change or a change in calibrators or reagent lot. Two levels of controls are also available for monitoring the cutoff level: 3.75 ng/mL and 6.25 ng/mL.

Calibration and Quality Control

Good laboratory practices recommend the use of at least two levels of control specimens (one positive and one negative control near the cutoff) to ensure proper assay performance. Controls should be run with each new calibration and after specific maintenance or troubleshooting procedures as detailed in the instrument system manual. Each laboratory should establish its own control frequency. If any trends or sudden change in control value are observed, review all operating parameters, or contact LZI technical support for further assistance. Laboratories should comply with all federal, state, and local laws, as well as all guidelines and regulations.

Results

Note: A preliminary positive test result does not necessarily mean a person took a specific drug and a negative test result does not necessarily mean a person did not take a specific drug. There are a number of factors that influence the reliability of drug tests.

Semi-Quantitative: The semi-quantitative mode is for purposes of (1) enabling laboratories to determine an appropriate dilution of the specimen for verification by a confirmatory method such as GC/MS, LC/MS or (2) permitting laboratories to establish quality control procedures. When an approximation of concentration is required, a calibration curve can be established with five calibrators. The concentration of norfentanyl in the sample may then be estimated from the calibration curve.

Limitations

1. *Boric Acid at 1% w/v may cause false negative results. Boric Acid is not recommended as a preservative for urine.*
2. *Dextromethorphan may cause false positive results at concentrations greater than 25,000 ng/mL.*
3. *A preliminary positive result from this assay indicates only the presence of norfentanyl and does not necessarily correlate with the extent of physiological and psychological effects (e.g., intoxication). This test is not intended for quantifying the individual analytes in samples.*
4. *A negative result does not necessarily mean a person did not abuse drugs.*
5. *Care should be taken when reporting results, as numerous factors (e.g., fluid intake, endogenous or exogenous interferents) may influence the urine test result.*
6. *Preliminary positive results should be confirmed by other affirmative, analytical methods (e.g., chromatography), preferably GC/MS or LC/MS.*
7. *The test is designed for use with human urine only.*
8. *The test is not for therapeutic drug monitoring.*

Typical Performance Characteristics

The results shown below were performed with a single Beckman Coulter AU680 automated chemistry analyzer.

Precision:

Semi-quantitative analysis: The following concentrations were determined with reference curves from 5 calibrators. Typical results were measured in ng/mL.

Concentration	Within Run (N=20)			Total Precision (N=80)		
	Mean	SD	% CV	Mean	SD	% CV
0 ng/mL	0.0	0.2	N/A	0.0	0.2	N/A
1.25 ng/mL	1.4	0.2	13.6 %	1.4	0.2	15.8 %
2.5 ng/mL	2.6	0.2	6.1 %	2.6	0.2	8.4 %
3.75 ng/mL	3.8	0.2	5.3 %	3.8	0.2	6.3 %
5 ng/mL	5.2	0.2	3.5 %	5.2	0.2	4.6 %
6.25 ng/mL	6.4	0.2	3.6 %	6.4	0.3	4.9 %
7.5 ng/mL	8.0	0.3	3.3 %	8.0	0.3	3.7 %
8.75 ng/mL	9.2	0.2	2.0 %	9.2	0.3	2.8 %
10 ng/mL	10.2	0.4	3.8 %	10.2	0.5	4.4 %

5 ng/mL Cutoff		Within Run (N=22)		Run-to-Run (N=88)	
Concentration	% of Cutoff	# Samples	EIA Result	# Samples	EIA Result
0 ng/mL	-100.0 %	22	22 Neg	88	88 Neg
1.25 ng/mL	-75.0 %	22	22 Neg	88	88 Neg
2.5 ng/mL	-50.0 %	22	22 Neg	88	88 Neg
3.75 ng/mL	-25.0 %	22	22 Neg	88	88 Neg
5 ng/mL	100.0 %	22	3 Neg/ 19 Pos	88	24 Neg/ 64 Pos
6.25 ng/mL	+25.0 %	22	22 Pos	88	88 Pos
7.5 ng/mL	+50.0 %	22	22 Pos	88	88 Pos
8.75 ng/mL	+75.0 %	22	22 Pos	88	88 Pos
10 ng/mL	+100.0 %	22	22 Pos	88	88 Pos

Accuracy: One-hundred and one (101) unaltered clinical urine specimens were tested with the LZI Fentanyl (Semi-Quantitative) Enzyme Immunoassay and confirmed by LC/MS. Specimens having a norfentanyl concentration greater than 5 ng/mL by LC/MS are defined as positive, and specimens with norfentanyl concentrations below 5 ng/mL by LC/MS are defined as negative in the table below. Near cutoff samples are defined as ±50 % of the cutoff value. The correlation results are summarized as follows:

Semi-Quantitative Accuracy Study:

5 ng/mL Cutoff	Neg	< 50 % of the cutoff	Near Cutoff Neg	Near Cutoff Pos	High Pos	% Agree-ment
Positive	0	1*	6**	8	41	100.0 %
Negative	21	19	5	0	0	86.5 %

The following table summarizes the result for the discordant samples:

5 ng/mL Cutoff	Norfentanyl LC/MS (ng/mL)	LC/MS	LZI EIA (ng/mL)	LZI EIA
38*	1.5	Neg	6.1	Pos
44*	3.0	Neg	6.0	Pos
46**	3.3	Neg	9.6	Pos
47**	3.5	Neg	14.0	Pos
48**	3.8	Neg	18.5	Pos
50**	4.2	Neg	9.6	Pos
52**	4.6	Neg	15.7	Pos

Specificity: Various potentially interfering substances were tested for cross-reactivity with the assay. Test compounds were spiked into the drug-free urine calibrator matrix to various concentrations and evaluated against the cutoff calibrator.

The following table lists the concentration of each test compound that gave a response approximately equivalent to that of the cutoff calibrator (as positive) or the maximal concentration of the compound tested that gave a response below the response of the cutoff calibrator (as negative). Compounds tested at high concentration with results below the cutoff value were listed as Not Detected (ND).

Fentanyl and Metabolites:

Compound	Concentration Tested (ng/mL)	% Cross-Reactivity	Result
Fentanyl	3.2	156.25 %	Positive
Norfentanyl	5	100.00 %	Positive

Structurally Related Compounds:

Compound	Concentration Tested (ng/mL)	% Cross-Reactivity	Result
4-Fluoro-isobutyryl Fentanyl	35	14.29 %	Pos
9-HydroxyRisperidone	100,000	0.01 %	Neg
Acetyl Fentanyl	7	71.43 %	Pos
Acetyl Norfentanyl	100	5.00 %	Pos
Acryl Fentanyl	3.5	142.86 %	Pos
Alfentanil	100,000	0.01 %	Neg
Butyryl Fentanyl	3.5	142.86 %	Pos
Butyryl Norfentanyl	35	14.29 %	Pos
Carfentanil Oxalate	100,000	0.01 %	Neg
Cis-d, I 3-Methylfentanyl	8.5	58.82 %	Pos
Cyclopropyl Norfentanyl	20	25.00 %	Pos
Despropionylfentanyl (4-ANPP)	100,000	0.01 %	Neg
Furanyl Fentanyl	6	81.97 %	Pos
Furanyl Norfentanyl	180	2.78 %	Pos
(±)-β-Hydroxythiofentanyl	5	100.00 %	Po
Isobutyryl Fentanyl	20	25.00 %	Pos
Isobutyryl Norfentanyl	400	1.25 %	Pos
Labetalol Hydrochloride	100,000	0.01 %	Neg
Methoxyacetyl Fentanyl	3.5	142.86 %	Pos
MT-45	100,000	0.01 %	Neg
N-benzyl Furanyl Norfentanyl	12	41.67 %	Pos
N-benzyl para-fluoro Norfentanyl	4.2	119.05 %	Pos
Norcarfentanil Oxalate	100,000	0.01 %	Neg
Ocfentanil	3.5	142.86 %	Pos
Para-fluorobutyryl Fentanyl (P-FBF)	5.5	90.91 %	Pos
para-Fluorofentanyl	3.1	163.93 %	Pos
Remifentanil	100,000	0.01 %	Neg
Risperidone	100,000	0.01 %	Neg
Sufentanil	100,000	0.01 %	Neg
Thienyl Fentanyl	3.5	142.86 %	Pos
Thiofentanyl	3.2	156.25 %	Pos
Trans-d, I 3-Methylfentanyl	6	83.33 %	Pos
Trazodone	100,000	0.01 %	Neg
U-47700	100,000	0.01 %	Neg
Valeryl Fentanyl	95	5.26 %	Pos
ω-1-Hydroxy Fentanyl	320	1.56 %	Pos

Structurally Unrelated Compounds:

Compound	Spiked [ ] (ng/mL)	Spiked Norfentanyl Concentration		
		0 ng/mL	3.75 ng/mL Control	6.25 ng/mL Control
(1S,2S)-(+)Pseudoephedrine	100,000	ND	Neg	Pos
6-Acetylmorphine	10,000	ND	Neg	Pos
Acetaminophen	100,000	ND	Neg	Pos
Acetylsalicylic Acid	100,000	ND	Neg	Pos
Amitriptyline	100,000	ND	Neg	Pos
Amlodipine Besylate	100,000	ND	Neg	Pos
Amoxicillin	100,000	ND	Neg	Pos
Atorvastatin	20,000	ND	Neg	Pos
Benzoylecgonine	100,000	ND	Neg	Pos
Buprenorphine	100,000	ND	Neg	Pos
Bupropion	100,000	ND	Neg	Pos
Caffeine	100,000	ND	Neg	Pos
Carbamazepine	100,000	ND	Neg	Pos
Cetirizine	100,000	ND	Neg	Pos
Chlorpheniramine	100,000	ND	Neg	Pos
Chlorpromazine	100,000	ND	Neg	Pos
Clomipramine	100,000	ND	Neg	Pos
Codeine	100,000	ND	Neg	Pos
d-Amphetamine	100,000	ND	Neg	Pos
Desipramine	100,000	ND	Neg	Pos
Dextromethorphan	40,000	Pos	Pos	Pos
Diphenhydramine	100,000	ND	Neg	Pos
d-Methamphetamine	100,000	ND	Neg	Pos
Duloxetine	100,000	ND	Neg	Pos
Fluoxetine	100,000	ND	Neg	Pos
Fluphenazine	100,000	ND	Neg	Pos
Gabapentin	100,000	ND	Neg	Pos
Hydrocodone	100,000	ND	Neg	Pos
Hydromorphone	100,000	ND	Neg	Pos
Ibuprofen	100,000	ND	Neg	Pos
Imipramine	100,000	ND	Neg	Pos
Lisinopril	100,000	ND	Neg	Pos
Loratadine	100,000	ND	Neg	Pos
Losartan	10,000	ND	Neg	Pos
l-Thyroxine	10,000	ND	Neg	Pos
MDA (3,4-methylenedioxyamphetamine)	100,000	ND	Neg	Pos
MDEA	100,000	ND	Neg	Pos
MDMA (3,4-methylenedioxy-methamphetamine)	100,000	ND	Neg	Pos
Meperidine	100,000	ND	Neg	Pos
Metformin	100,000	ND	Neg	Pos
Methadone	100,000	ND	Neg	Pos
Metoprolol	100,000	ND	Neg	Pos
Morphine	100,000	ND	Neg	Pos
Nicotine	100,000	ND	Neg	Pos
Nortriptyline	100,000	ND	Neg	Pos
Omeprazole	100,000	ND	Neg	Pos
Oxazepam	100,000	ND	Neg	Pos
Oxycodone	100,000	ND	Neg	Pos
Oxymorphone	100,000	ND	Neg	Pos
Phencyclidine (PCP)	100,000	ND	Neg	Pos
Phenobarbital	100,000	ND	Neg	Pos
Quetiapine	100,000	ND	Neg	Pos
Ranitidine	100,000	ND	Neg	Pos
Salbutamol (Albuterol)	100,000	ND	Neg	Pos
Sertraline	100,000	ND	Neg	Pos
THC-COOH (11-Nor-Delta-9-THC-9-carboxylic acid)	100,000	ND	Neg	Pos
Tramadol	100,000	ND	Neg	Pos
Zolpidem	10,000	ND	Neg	Pos

It is possible that other substances and/or factors not listed above may interfere with the test and cause false positive results.

The following structurally unrelated compound which showed interference at ±25 % of cutoff concentrations was then spiked into pooled negative human urine at ±50 % of cutoff concentrations (2.5 ng/mL and 7.5 ng/mL) for the assay. Interference was still observed with dextromethorphan. Results are summarized in the following table:

Compound	Spiked [ ] (ng/mL)	Spiked Norfentanyl Concentration		
		0 ng/mL	2.5 ng/mL	7.5 ng/mL
Dextromethorphan	40,000	Pos	Pos	Pos

Endogenous and Preservative Compound Interference Study:
The following endogenous compounds were spiked into pooled negative human urine and the two levels of controls (3.75 ng/mL and 6.25 ng/mL) for the assay. The spiked solution was evaluated against cutoff calibrator. Interference was observed with Boric Acid. No other major interference with these compounds at physiological relevant concentrations as all spiked samples gave correct corresponding preliminary positive/negative results against the cutoff value of 5 ng/mL. Results are summarized in the following table:

Endogenous Substance	Spiked [ ] (mg/dL)	Spiked Norfentanyl Concentration		
		0 ng/mL	3.75 ng/mL Control	6.25 ng/mL Control
Acetone	1000	Neg	Neg	Pos
Ascorbic Acid	1500	Neg	Neg	Pos
Bilirubin	2	Neg	Neg	Pos
Boric Acid	1000	Neg	Neg	Neg
Calcium Chloride (CaCl2)	300	Neg	Neg	Pos
Citric Acid (pH 3)	800	Neg	Neg	Neg
Creatinine	500	Neg	Neg	Pos
Ethanol	1000	Neg	Neg	Pos
Galactose	10	Neg	Neg	Pos
γ-Globulin	500	Neg	Neg	Pos
Glucose	3000	Neg	Neg	Pos
Hemoglobin	300	Neg	Neg	Pos
β-hydroxybutyric Acid	100	Neg	Neg	Pos
Human Serum Albumin	500	Neg	Neg	Pos
Oxalic Acid	100	Neg	Neg	Pos
Potassium Chloride	6000	Neg	Neg	Neg
Riboflavin	7.5	Neg	Neg	Pos
Urea	6000	Neg	Neg	Pos
Uric Acid	10	Neg	Neg	Pos
Sodium Azide	1000	Neg	Neg	Pos
Sodium Chloride	6000	Neg	Neg	Pos

The following endogenous compounds which showed interference at ±25 % of cutoff concentrations were then spiked into negative urine and at ±50 % of cutoff concentrations (2.5 ng/mL and 7.5 ng/mL) for the assay. Interference was still observed with Boric Acid at 1 % w/v. Results are summarized in the following table:

Endogenous Substance	Spiked [ ] (mg/dL)	Spiked Norfentanyl Concentration		
		0 ng/mL	2.5 ng/mL	7.5 ng/mL
Boric Acid	1000	Neg	Neg	Neg
Citric Acid (pH 3)	800	Neg	Neg	Pos
Potassium Chloride	6000	Neg	Neg	Pos

pH Interference Study: Negative urine and urine spiked with analyte to the two levels of controls (3.75 ng/mL and 6.25 ng/mL) were adjusted to the following pH levels and tested by the assay. The pH adjusted solutions were evaluated against the cutoff calibrator.
No major interference with these pH levels was observed as all pH adjusted levels gave correct corresponding preliminary positive/negative results against the cutoff value of 5 ng/mL. Results are summarized in the following table:

pH	Spiked Norfentanyl Concentration		
	0 ng/mL	3.75 ng/mL Control	6.25 ng/mL Control
pH 3	Neg	Neg	Pos
pH 4	Neg	Neg	Pos
pH 5	Neg	Neg	Pos
pH 6	Neg	Neg	Pos
pH 7	Neg	Neg	Pos
pH 8	Neg	Neg	Pos
pH 9	Neg	Neg	Pos
pH 10	Neg	Neg	Pos
pH 11	Neg	Neg	Pos

Specific Gravity: Samples ranging in specific gravity from 1.003 to 1.028 were split into three portions each and either left un-spiked or further spiked to a final norfentanyl concentration of either 3.75 ng/mL or 6.25 ng/mL (the negative and positive control concentrations, respectively). These samples were then evaluated in qualitative mode. No interference was observed.

Open-Vial Reagent and Calibrator/Control Stability: Real-time data for open-vial reagent and calibrator/control stability studies at Cold Temperature (2-8ºC) have been carried out up to Day 736. Results from open-vial studies indicate that degradation is minimal up to Day 736, and, based on the real-time data, suggests an open-vial stability of up to 24 months. Open-vial reagents and calibrators/controls should be stored at 2-8ºC for maximum shelf life.

Closed-Vial Calibrator/Control Stability: Real-time data for closed-vial calibrator/control stability studies at Cold Temperature (2-8ºC) have been carried out up to Day 736. Results from closed-vial studies indicate that degradation is minimal at Cold Temperature (2-8ºC) up to Day 736 in comparison to Day 1. Closed-vial calibrators/controls should be stored at 2-8ºC for maximum shelf life.

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Additions, deletions, or changes are indicated by a change bar in the margin.

For technical assistance please call: (408) 970-8811

Manufacturer:
Lin-Zhi International, Inc.
2945 Oakmead Village Court
Santa Clara, CA 95051
USA
Tel: (408) 970-8811
Fax: (408) 970-9030
www.lin-zhi.com